PhD theses

Stress tolerance of transgenic barley plants expressing aldo-keto reductase genes

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Introduction

Plants are exposed to oxidative damage as secondary stress, under various stress conditions. A major proportion of oxidative stress originates from the photosynthetic system. For instance, dark reactions of the photosythesis can be suppressed, either as a consequence of low stomatal conductance (caused by water deficit or Cadmium treatment) or of low temperature. In these cases, light could become excess and reactive oxygen species (ROS) are formed during light reactions. These compounds not only contribute to injury of different parts of the cell *per se*, but cause indirect injuries via products of lipid peroxidation. Lipid peroxidation refers to the reaction of ROS with membrane lipids. Certain lipid peroxide breakdown products, the reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) contain two double bonds and so are able to cross-link proteins. These compounds are effectively detoxified in many plants including e.g. alfalfa, rice and corn by stress-inducible aldo-keto reductase enzymes (AKRs). The expression of these proteins may improve tolerance against drought (Oberschall *et al.* 2000), heavy metals (Hegedüs *et al.* 2004), UV (Hideg *et al.* 2003) and heat (Turóczy *et al.* 2011). The AKR4C9 enzyme of *Arabidopsis thaliana* origin has also been shown to be active on reactive aldehyde substrates (Simpson *et al.* 2009). **Based on these data, our research focused on AKR4C9. With regards to its reactive aldehyde detoxifying function, the following research questions have been formulated:**

- **What is the distribution of the AKR-mediated detoxification mechanism in the plant kingdom?**
- **Does AKR4C9 contribute to heavy metal tolerance of the plants, similarly to the alfalfa enzyme?**
- **How does the expression of this enzyme alter the reactive aldehyde detoxifying capacity?**
- **Is it possible to adapt a cheap plant physiology test for characterising the reactive aldehyde detoxifying capacity of the plants *in vivo*? If so, what plant physiological and growth parameters can be identified as specific effects of reactive aldehydes on the plant?**

Osmotic stress as secondary stress also presents a common threat during many types of environmental stresses, including at least drought, freezing, and salt stress. During these

conditions, plant cells may be exposed to dehydration and protein denaturation. The production of osmoprotectants e.g. sugar alcohols may improve the osmotic stress tolerance by providing osmoprotection and osmotic adjustment. On one hand, many AKRs are known to be capable of sugar alcohol production, including e.g. the celery mannose-6-phoshate reductase. The celery enzyme has been ectopically expressed in transgenic Arabidopsis, and it lead to mannitol production and conferred to enhanced salt stress tolerance (Zifang and Loescher, 2003). On the other hand, less evidence exists for that some AKRs can both detoxify reactive aldehydes and synthesise sugar alcohols. Sugar alcohol production can ameliorate not only salinity, but freezing tolerance as well. **With regards to sugar alcohol production of AKRs, the following issues were intended to be resolved:**

- **Is a reactive aldehyde detoxifying AKR-enzyme capable of sugar alcohol synthesis? In that case, which molecule is the source?**
- **How does AKR4C9 affect salinity and freezing stress tolerance of transgenic plants?**

In order to collect these missing data, transgenic barley plants have been studied constitutively over-expressing AKR4C9. These transgenic lines have already been established (Éva *et al.* 2008). During the PhD thesis work, transgene copy number has been determined. Besides this, the activity of AKR4C9 (purified from transgenic barley plants) has been studied against carbohydrate and reactive aldehyde type substrates, both *in vivo* and *in vitro*. The level of sorbitol, a possible product of AKR4C9 has also been analysed, but within the plants. Furthermore, tolerance of both transgenic and non-transgenic barley plants have been assessed during abiotic stress conditions generating strong oxidative and/or osmotic stress.

Materials and methods

Plant material: the transgenic barley (*Hordeum vulgare* L. cv. 'Golden Promise') plants (T3 progeny generation) carried the rice actin promoter :: *Arabidopsis thaliana At2g37770.2* gene :: *Agrobacterium tumefaciens nos* terminator construct. Potted plants (e.g. for enzyme extraction and raising progeny generations) were grown in a 3:1 mix of peat-containing soil and sand. The plants were grown in a SANYO Phytotron growth chamber at 70 % humidity, with a 16 hour photoperiod and light intensity of 200 μmol/m^2•s, 15 °C day and 12 °C night temperature. Hydroponically grown barley plants (for plant physiology

experiments) were were grown in ¼ strength Hoagland solution under a slightly modified protocol of Fodor *et al.* (1998) in a Phytotron chamber providing a 14 hour photoperiod (light intensity of 200 µmolm $^{-2}$ s^{-1}) at a day/night temperature regime of 24/18°C.

Copy number determination: Transgene copy number was estimated by quantitative real-time PCR (q-PCR), using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2001). Q-PCR was performed for the *At2g37770.2* transgene, and the single copy barley *Blz2* gene (Gene Bank accession number: Y10834.1) was used as normaliser.

Enzime kinetics: recombinant, His-tag fusion AKR4C9 enzyme has been purified from transgenic barley leaves by affinity chromatography. The *in vitro* enzyme activity measurements for the AKR enzyme were performed according to Vander Jagt *et al.* (1992), following the absorbance of cofactor NADPH.

Determination of sugar alcohol content: sorbitol content of the plants was analysed by HPLC-RID (high performance liquid chromatography with refractive index detector), using the modified method of Simonzadeh and Ronsen (2012). The analysis was carried out in co-operation by Rita Tömösközi-Farkas in the Research and Innovation Center, Food Science Research Institute, Budapest (NAIK, formerly KÉKI).

Stress-physiology experiments: have been carried out on hydroponically-grown plants. Direct reactive aldehyde tolerance has been assessed by spraying twice during a 3-day treatment with a glutaraldehyde solution of 0.1 V/V %. Cadmium tolerance has been studied by applying 5 days of 10 µM $Cd(NO_3)_2$ treatment, while salt tolerance has been studied by applying 6 days of 175 mM NaCl treatment. Both Cadmium-nitrate and sodium-chloride has been included in the hydroponic solution. The level of freezing tolerance has been analysed by applying three subsequent freeze-thawing at -20 °C, after 4 days of cold acclimation at 4 °C.

Determination of photosynthetic pigment content: chlorophyll contents were determined in 80 % (v/v) acetone extracts using an UV–VIS spectrophotometer (Shimadzu) according to Porra *et al.* (1989). Total carotenoid content was determined from 100% (v/v) acetone extracts by an UV-VIS spectrophotometer (Shimadzu) according to Lichtenhaler *et al.* (1987).

Fluorescence induction measurements of leaf samples were carried out as described by Solti *et al.* (2008), using a PAM 101–102–103 chlorophyll fluorometer (Walz, Effeltrich, Germany).

Malondialdehyde (MDA) content of leaves was determined as described by (Kovács *et al.* 2009).

Electrolyte leakage test: the percentage of electrolyte leakage was measured to evaluate membrane injury, using the method of Hara *et al.* (2003). It was determined on leaf area basis. Leaves were immersed in 10 ml deionised water for 1.5 h at room temperature. After incubation conductivity of the solution was measured using a conductivity meter (Radelkis OK 112, Budapest, Hungary). Total electrolyte leakage was determined after boiling untreated samples for 15 min and cooling to room temperature. Electrolyte leakage is given as a percentage of total electrolyte leakage.

Statistical analysis: five independent experiments were carried out to study each physiological parameter. Variance analysis or unpaired T-test were performed on the data. Graphpad Instat and Microcal Origin were used for statistical analysis.

Results and discussion

1. The transgene copy number ranged from 1 to 4. These low values are typical for agrobacterium-mediated transformation.

2. Enzyme activity of AKR4C9 has been detected against substrates glutaraldehyde and fructose *in vitro*. Based on this, it was assumed that reactive aldehyde detoxifying AKRs may synthesise sugar alcohols by reducing sugars. Besides this, glutaraldehyde has emerged as a candidate for studying the reactive aldehyde detoxifying capacity of intact plants, *in vivo*.

3. Reactive aldehyde detoxifying function of AKR4C9 was also studied *in vivo* by administering glutaraldehyde spraying. The AKR4C9-expressing transgenic plants showed higher reactive aldehyde detoxifying capacity, based on many growth and physiological parameters including fresh weight, chlorophyll-content, fluorescence induction parameters and respiratory rate. It was the first time that the effects of a reactive aldehyde molecule has

been characterised in intact plants with relation to growth and physiological parameters. Only leaf-disc assays have been applied for this purpose so far.

4. The high AKR4C9 expressing line C1 possessed much lower malondialdehyde content during Cadmium stress, than the wild type. Furthermore, its total chlorophyll content and PSII maximal quantum efficiency (F_v/F_m) was found to be significantly higher. Altogether, AKR4C9 has enhanced the Cadmium tolerance of the plants, but to a low extent. It is of no surprise however, because AKRs clearly cannot protect the plants from all effects of cadmium (e.g. protein denaturation, inhibition of iron uptake).

5. The sugar alcohol, sorbitol could be detected in both transgenic and non-transgenic plants, though it reached 2-4-fold higher level in transgenics. These measurements confirmed sorbitol production of AKR4C9, with fructose as a possible source.

6. In the next set of experiments, transgenic and non-transgenic plants have been exposed to stresses generating osmotic stress, where sorbitol production may provide an advantage. Salt stress tolerance was studied by measuring total chlorophyll content, chlorophyll *a/b* ratio, F_v/F_m parameter and chlorophyll/carotenoid ratio. These measurements have indicated considerably higher salinity tolerance of the transgenic plants. It was solely the result of sugar alcohol production, since malondialdehyde content did not increase in any plants. The lack of increase in MDA content argues for the non-existance of strong oxidative stress during our salt treatment. As a consequence, not the antioxidant function, but rather osmoprotective function of sorbitol could have enhanced the salt tolerance.

7. As for freezing treatments, it was remarkable that more leaves of the high AKR4C9-expressing line C1 has survived the freeze-thaw cycles at -20°C, than of non-transgenic or low expression level plants. Line C1 plants also exhibited lower average electrolyte leakage and regenerated faster after the freezing. Mild lipid peroxidation has been observed in all frost-treated plants, though there was no difference between transgenic and non-transgenic plants. It is possible, that AKR4C9 has low activity at low temperature, but facilitates the recovery of frost treated plants at higher temperature, by removing reactive aldehydes. Higher regenerative capacity of AKR4C9-expressing plants has been confirmed by our measurements. Nevertheless, increased sorbitol production was suggested in our study as the most important protective mechanism against freezing. Sorbitol as osmoprotectant may present an immediate defence.

Some of the most remarkable results and conclusions

- **Glutaraldehyde spraying is suitable for testing the reactive aldehyde detoxifying capacity of plants *in viv*o, though it causes growth retardation even in the highly tolerant ones.**
- **Similarly to the previously described MsALR of alfalfa origin, the Arabidopsis enzyme AKR4C9 has also enhanced Cadmium tolerance of transgenic plants.**
- **The first clear, *in vivo* evidence is provided for sugar alcohol production of a reactive aldehyde reducing AKR.**
- **The expression of AKR4C9 lead to higher salinity and frost tolerance of transgenic plants.**

I. The applied glutaraldehyde test is a cheap and simple one, being suitable for getting information on the reactive aldehyde neutralizing capacity of different plant species or varieties. It may predict their abiotic stress tolerance as well. The transgene *At2g37770.2* provided a high-level tolerance against the direct reactive treatment as indicated by the high chlorophyll content maintained by the high expressing line C1. However plants in this line still showed signs of growth arrest, since unlike untreated controls the treated plants could not reach 4-leaf-stage. Based on these findings, signalling function of reactive aldehydes can be considered. In human and animal cells, 4-HNE, a diffusible reactive aldehyde, is known to be involved in signalling through the MAP kinase pathway. The signal may lead to induction or arrest of cell growth depending on the concentration of 4-HNE as well as the age and type of the cell. This phenomenon has not been described in the plant kingdom yet, and may serve as focus of future research projects.

II. Based on our results, all stress inducible aldo-keto reductase enzymes may play a rolet in heavy metal tolerance. On one hand according to the literature, reactive aldehyde removal may enhance Cadmium tolerance. On the other hand, sorbitol production of AKR4C9 could have also contributed, since sugar alcohols are known as antioxidants.

III. With regards to sugar alcohol production the transgene *At2g37770.2* proved to be more useful, than other transgenes previously applied for this purpose. This statement is based on that transgenes applied, e.g. the bacterial mannitol-1-phosphate dehydrogenase did not offer any further advantages for the plant according to the literature. In contrast the aldo-keto reductase enzyme studied in this project was even able to reduce harmful reactive aldehydes over sugar alcohol production and therefore present an other benefit. Thanks to these functions transgenic plants can be simultaneously protected against both oxidative and osmotic stresses.

IV. Other AKRs, whose primary function is the production of sugar alcohols and not the reduction of reactive aldehydes have been studied more extensively in terms of salinity. However we provided evidence for that reactive aldehyde detoxifying AKRs may also enhance salt tolerance due their other function, the sugar alcohol production. This PhD. thesis work includes the deepest analysis for the role of any AKRs in freezing tolerance. We assume, that both function of AKRs are involved in freezing stress tolerance. Up to date, only one study has indicated the role of an AKR enzyme in freezing tolerance (Lee and Chen, 1993). They induced freezing tolerance of bromegrass (*Bromus inermis*) cell suspension by applying abscisic acid treatment. Among the abscisic acid-responsive genes an aldose-reductase gene was identified which may therefore be associated with freezing stress tolerance (Lee and Chen, 1993).

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B. Conference abstracts:

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